Glutathione Concentration during Maturation and after Fertilization in Pig Oocytes: Relevance to the Ability of Oocytes to Form Male Pronucleus[1]

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ABSTRACT

The present study examined the kinetics of glutathione (GSH) concentration during maturation and after fertilization in pig oocytes and its relevance to the ability of pig oocytes to form a male pronucleus after in vitro fertilization. The GSH concentration was significantly higher in pig oocytes matured in Waymouth medium than in pig oocytes matured in either modified (m) TCM-199 or mTLP media. The addition of 0.04–0.57 mM cysteine (CySH) to mTLP significantly increased both the GSH concentrations in oocytes matured in vitro and the rate of male pronucleus formation as compared to those in oocytes cultured in mTLP alone. When pig oocytes were cultured 12, 24, or 36 h in mTLP plus 0.14 mM CySH, their GSH concentrations were significantly higher than in uncultured oocytes. After fertilization, the GSH concentration in pig oocytes declined significantly. GSH concentrations in oocytes matured in vivo did not differ from those in oocytes matured in mTLP plus 0.14 or 0.57 mM CySH. The results indicate that 1) the composition of maturation medium affects the GSH concentration in pig oocytes; 2) the addition of CySH to maturation medium permits GSH synthesis by the pig oocytes; 3) GSH levels in pig oocytes change during maturation and after fertilization; and 4) GSH synthesis during oocyte maturation is an important factor for promoting their ability to form a male pronucleus after fertilization.

INTRODUCTION

Studies on the cellular and molecular factors operating during oocyte maturation and fertilization provide the basis for defining conditions for the production of embryos in vitro and their possible application in basic research and animal breeding.

Glutathione (r-glutamylcysteinylglycine; GSH) is a major intracellular free thiol that has important biological functions during cellular proliferation, amino acid transport, synthesis of protein and DNA, and reduction of disulfides and other chemicals; and it protects cells against oxidation [1, 2]. The synthesis of GSH during oocyte maturation is reported to be a prerequisite for sperm chromatin decondensation and hence for male pronucleus formation after sperm penetration of mouse [3] and hamster oocytes [4, 5].

The ability of pig oocytes to mature, be fertilized, and develop in vitro has been demonstrated by several investigators [6–8]. However, one major problem that persists is a high incidence of failure of the sperm to form a functional male pronucleus after it has penetrated the oocyte [6–11]. In a previous study, we found that the composition of maturation media had a major influence on whether the male pronucleus forms [12]. A high concentration of cysteine (CySH) in the medium was identified as a major fac-

tor, and we suggested that CySH possibly served as a substrate for GSH.

The objective of the study reported here was to determine the kinetics of GSH concentration in pig oocytes during maturation in medium containing different amounts of CySH, and after fertilization in relation to the ability of pig oocytes to form a male pronucleus after in vitro fertilization.

MATERIALS AND METHODS

The methods for in vitro maturation and fertilization of oocytes were based on those described by Yoshida et al. [12].

Collection of Oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl containing 100 mg kanamycin sulfate/l (Meiji Seika, Tokyo, Japan) at 35°C. Within 2 h of slaughter, the follicular contents were recovered from the follicles (2–5 mm in diameter) by aspiration with a 21-gauge needle (Terumo Co., Tokyo, Japan) and a 5-ml disposable syringe (Nipro, Osaka, Japan) . The oocyte-cumulus complexes were gathered from the follicular contents and washed twice with Hepes-TALP (tyrode-albumin-lactate-pyruvate)-PVA (polyvinylalcohol) [13] and the maturation medium, respectively. Only oocytes possessing a compact cumulus mass and evenly granulated ooplasm were selected for the experiments.

Prepubertal gilts (crossbred) were superovulated with 1250 IU eCG (Teikoku Zoki Co., Tokyo, Japan) and 500 IU hCG (Sankyo Co., Tokyo, Japan) at 72-h intervals as described by Yoshida [14], and the in vivo-matured oocytes were collected 40–44 h after the last hCG injection.

Preparation of Spermatozoa

The sperm-rich fraction of ejaculates was obtained from a Large White boar by means of the gloved-hand method. Semen samples were washed three times with 0.9% (w/v) NaCl containing 100 mg BSA/l (Sigma Chemical Co., St Louis, MO) and 100 mg kanamycin sulfate/L. Washed spermatozoa were subsequently diluted to 50×10^6 cells/ml in the fertilization medium and used for insemination.

In Vitro Maturation and Fertilization

Oocytes (10–15) were transferred to a droplet of maturation medium (0.2 ml) under paraffin oil (Nakarai Tesque, Inc., Kyoto, Japan) in a polystyrene dish (35 mm: Becton Dickinson Labware, Oxnard, CA) and cultured. After incubation, oocytes (25–35) with an expanded cumulus mass were transferred to fertilization medium (2.0 ml) covered with paraffin oil. The spermatozoa were introduced into the fertilization medium to make a final concentration of $2.5–5 \times 10^4$ cells/ml.

Assessment for Maturation and Pronucleus Formation

Oocytes (12 h post-insemination) were fixed with acetic alcohol (methanol and acetic acid, 3:1, v/v) and stained with 1% aceto-orcein. The stages of oocytes were determined by means of Nomarski differential interference microscopy (Olympus, Tokyo, Japan): oocytes with a polar body were regarded as matured; matured oocytes with both a female pronucleus and an enlarged sperm head with sperm tail or both male (sperm tail) and female pronuclei were regarded as penetrated.

Culture Media and Culture Conditions

Three culture media were used for in vitro maturation of oocytes in this study: 1) modified (m) TCM-199 with Earle's salts (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.91 mM sodium pyruvate (Sigma), 3.05 mM glucose (Wako Pure Chemical Co., Osaka, Japan), 2.92 mM calcium lactate (Nakarai) as described by Yoshida et al. [12]; 2) Waymouth MB 725/1 (Sigma); and 3) mTLP-PVA supplemented with 1.0 mM glutamine, 0.2 mM isoleucine, 0.05 mM methionine, and 0.1 mM phenylalanine (all from Sigma) as described by Bavister [13]. The pH of each medium after equilibration with 5% CO_2 in air was 7.4. All maturation media were supplemented with 10 IU eCG/ml, 10 IU hCG/ml, 1 µg estradiol-17β/ml (Sigma), 100 µg kanamycin sulfate/ml, 10% (v/v) fetal calf serum (FCS; GIBCO, Grand Island, NY), and 10% (v/v) pig follicular fluid fraction (10 000 $< M_r < 200\,000$) [7, 8].

The fertilization medium consisted of 90% (v/v) mTCM-199 supplemented with 2 mM caffeine (Wako), 100 µg kanamycin sulfate/ml, and 10% (v/v) FCS. In vitro maturation and fertilization were carried out at 38.5°C in an atmosphere of 5% CO_2 in air. The handling of oocytes under the microscope was done at 38°C on a heated stage (Kitazato Supply Co., Fujinomiya, Japan).

Assay of GSH

After oocytes were incubated or collected, they were removed from the surrounding cumulus cells and/or spermatozoa by agitation with a narrow-bore glass pipette in Hepes-TALP-PVA and washed three times with PBS. Then groups of 10–30 oocytes in 5 µl of PBS were transferred to a 0.5-ml microfuge tube by means of a micropipette (Varipette 4810; Eppendorf, Hamburg, Germany) and frozen (−20°C). The frozen samples were thawed, 5 µl of 1.25 M H_3PO_4 (Wako) was added, and the oocytes were ruptured by agitation with a narrow-bore glass pipette and refrozen for storage. Blanks containing 5 µl of PBS without oocytes were similarly prepared.

GSH was determined by the enzymatic cycling assay of Tietze [15]. Briefly, the samples were thawed, diluted with 0.49 ml of H_2O, and transferred to a glass tube. The following solutions were then added to the tube: 0.7 ml of H_2O, 1.2 ml of 0.2 M potassium phosphate/10 mM EDTA buffer (pH 7.2), 0.1 ml of 10 µM 5,5-dithiobis-(2-nitrobenzonic acid) (Wako), 0.05 ml of 20 U/ml glutathione reductase (Oriental Yeast Co., Ltd., Osaka, Japan), and 0.05 ml of 4 µM NADPH (Kohjin Co., Ltd., Tokyo, Japan). Immediately after the addition of NADPH, the absorbance was monitored at 412 nm with a spectrophotometer (Hitachi Manufactory Co., Tokyo, Japan) and recorded at 0.5 and 5 min after the addition of NADPH. Both the reagent blank and GSH (Sigma) standards (0.1–1.0 nmol) were assayed under the same conditions. The amount of GSH in each sample was determined by comparison with a standard curve prepared at the same time. This amount was divided by the number of oocytes in the sample to obtain total GSH content per oocyte. To calculate the mean cell volume, a total of 110 oocytes were photographed by means of inverted microscopy (Nikon, Tokyo, Japan), and the diameter of the ooplasm was measured with a slide caliper. The mean diameter of the ooplasm in the oocytes was 118.5 ± 0.3 µm, and calculation by the equation $V = 4 \pi r^3/3$ indicated that the mean cell volume of the oocytes was 871.5 pl. Then, the GSH concentration per oocyte was estimated by determining total GSH content of oocytes and mean cell volume as described by Calvin et al. [3].

Statistics

Mean values are presented as mean ± SEM. GSH concentration was evaluated by Student's t-test. Chi-square analysis was used to compare the rate of maturation, sperm penetration, and male pronucleus formation in oocytes.
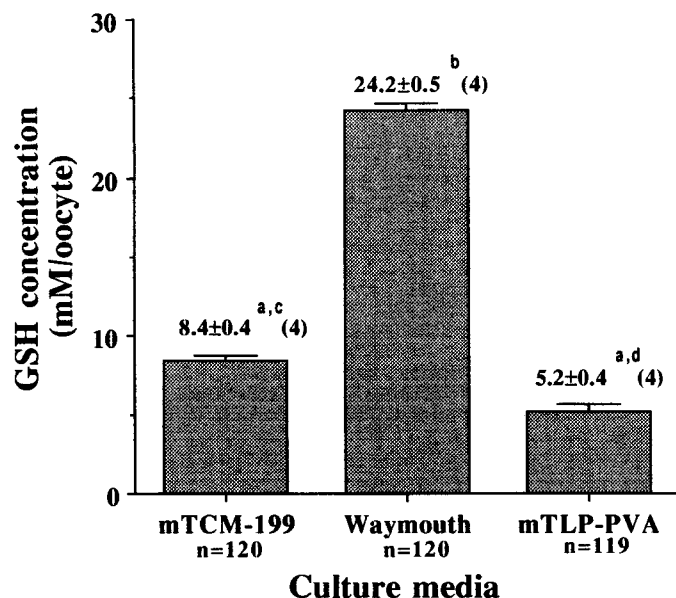
FIG. 1. Effect of maturation media on GSH concentration in pig oocytes matured for 36 h in vitro. The results represent the mean ± SEM of groups of oocytes collected in separate experiments. Numbers in parentheses indicate number of replicates. n, total number of oocytes assayed. $p < 0.01$ for values of a versus b and c versus d.
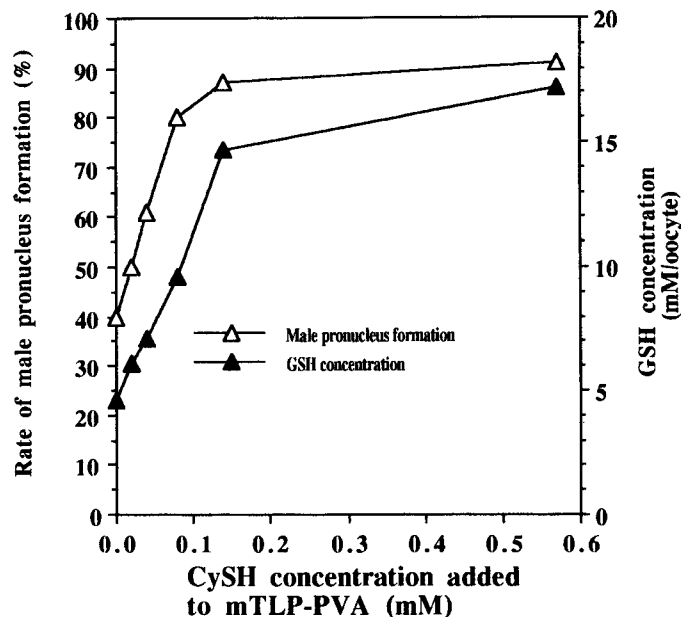

FIG. 2. Comparison of CySH effect on the rate of male pronucleus formation in pig oocytes matured and fertilized in vitro and on GSH concentration in pig oocytes matured in vitro. Data summarize the results represented in Table 1.

RESULTS

Experiment 1

The first experiment determined the effects of maturation media on GSH concentration in pig oocytes matured in vitro. Oocytes were cultured for 36 h in three maturation media before GSH concentration was assayed. As shown in Figure 1, the GSH concentration was significantly higher ($p < 0.01$) in oocytes matured in Waymouth medium than in those matured in mTCM-199 or mTLP. Also, the GSH concentration was significantly higher in oocytes matured in mTCM-199 than in those matured in mTLP ($p < 0.01$).

Experiment 2

The second experiment examined the effects of adding CySH to maturation medium on GSH concentration and male pronucleus formation in pig oocytes. Oocytes were cultured for 36 h in mTLP with or without 0.02–0.57 mM L-CySH (Sigma) before insemination. These concentrations were chosen to provide a wide range of CySH concentrations up to that found in Waymouth's medium. Some oocytes were randomly removed at 36 h of culture for GSH assay, and the remaining ones were inseminated in vitro to assess maturation and fertilization. As shown in Table 1, the rates of male pronucleus formation were significantly higher in oocytes matured in the media supplemented with as lit-

TABLE 1. Effect of cysteine (CySH) addition to mTLP-PVA on in vitro maturation, penetration, male pronucleus formation and GSH concentration in pig oocytes.

Addition of CySH (mM)	N[a]	No. (%) of oocytes			N[a]	GSH level; mean ± SEM/oocyte (No. of oocytes assayed)	
		Nuclear maturation	Sperm penetration[b]	Male pronucleus formation[c]		Total; pmol	Concentration; mM
control	8	193/214 (93)	175/193 (91)	70/175 (40)	4	4.0 ± 0.8 (120)	4.6 ± 0.9
0.02	3	48/56 (86)	44/48 (92)	22/44 (50)	4	5.3 ± 0.3 (120)	6.1 ± 0.4
0.04	5	91/103 (88)	81/91 (89)	49/81 (61)**	4	6.2 ± 0.4* (120)	7.1 ± 0.4*
0.08	6	123/142 (87)	112/123 (91)	89/112 (80)**	4	8.2 ± 0.2** (120)	9.6 ± 0.3**
0.14	6	137/158 (87)	119/137 (87)	103/119 (87)**	4	12.8 ± 0.8** (120)	14.7 ± 0.9**
0.57	4	73/89 (82)	67/73 (92)	61/67 (91)**	4	15.0 ± 0.3** (120)	17.2 ± 0.4**

[a]Number of replicates.
[b]In matured oocytes.
[c]In matured and penetrated oocytes.
*$p < 0.05$, **$p < 0.01$ compared with the control value.
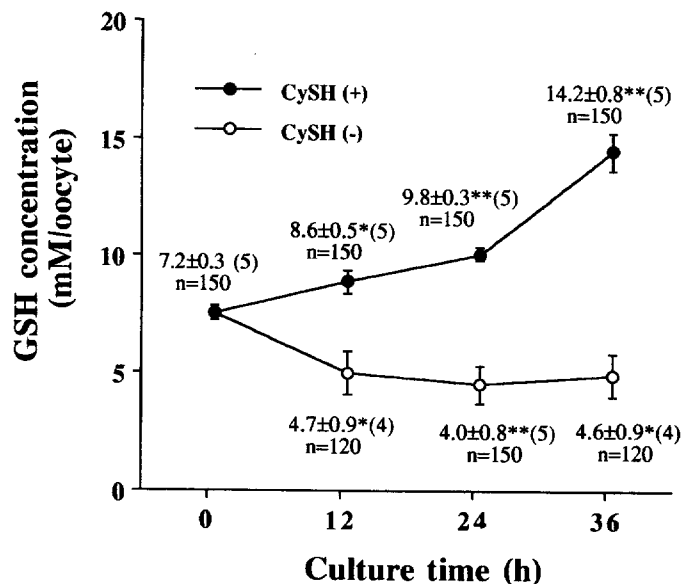
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FIG. 3. Effect of culture time on GSH concentration in pig oocytes cultured in mTLP supplemented with (+) or without (−) CySH in vitro. Results represent the mean ± SEM of groups of oocytes collected in separate experiments. Numbers in parentheses indicate number of replicates. n, total number of oocytes assayed. * $p < 0.05$, ** $p < 0.01$, compared with control (0 h of culture).
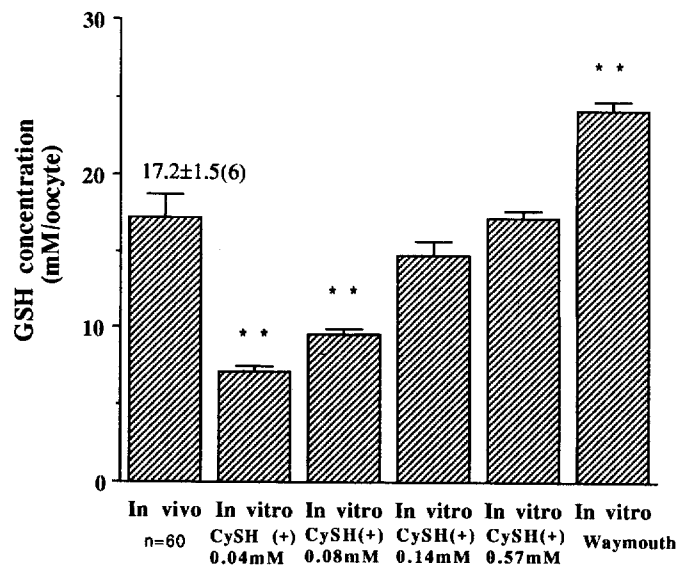

FIG. 4. Comparison of GSH concentrations in pig oocytes matured in vivo and those matured in different media in vitro. Data in the left bar are from experiment 4, those in the next 4 bars from experiment 2, and in the right bar from experiment 1. The results represent the mean ± SEM of groups of oocytes collected in separate experiments. Numbers in parentheses indicate number of replicates. n, total number of oocytes assayed. ** $p < 0.01$, compared with oocytes matured in vivo.

tle as 0.04 mM CySH in comparison to oocytes matured in control medium ($p < 0.01$), whereas the rates of nuclear maturation and sperm penetration of oocytes did not differ among media. Moreover, the GSH concentrations were significantly higher in oocytes matured in media containing as little as 0.04 mM CySH than in oocytes matured in the control medium ($p < 0.05$ or $p < 0.01$). It is evident from Figure 2 that addition of CySH to the maturation media caused a dose-dependent increase in both the rate of pronucleus formation and the GSH concentration. The maximal stimulatory effect was obtained with addition of 0.57 mM CySH.

Experiment 3

The third experiment examined the effect of culture time on GSH concentration in pig oocytes. Oocytes were cultured for 12–36 h in mTLP supplemented with or without 0.14 mM CySH before GSH concentrations were assayed. As shown in Figure 3, the concentration of GSH was significantly higher in oocytes by 12 h of culture in the CySH-supplemented medium in comparison to GSH concentration in oocytes before culture ($p < 0.01$). In addition, the GSH concentration continued to increase as the duration of culture increased. In contrast, the GSH concentration in pig oocytes cultured for 12–36 h in mTLP without CySH was significantly lower at each time point than in oocytes assayed immediately after collection ($p < 0.05$ or $p < 0.01$).

Experiment 4

The fourth experiment compared the GSH concentration of pig oocytes matured in vivo with that of pig oocytes

matured in vitro in mTLP supplemented with 0.04–0.57 mM CySH (experiment 2) and Waymouth medium (experiment 1). As shown in Figure 4, the GSH concentration was significantly higher in oocytes matured in vivo than in oocytes matured in mTLP supplemented with 0.04 or 0.08 mM CySH
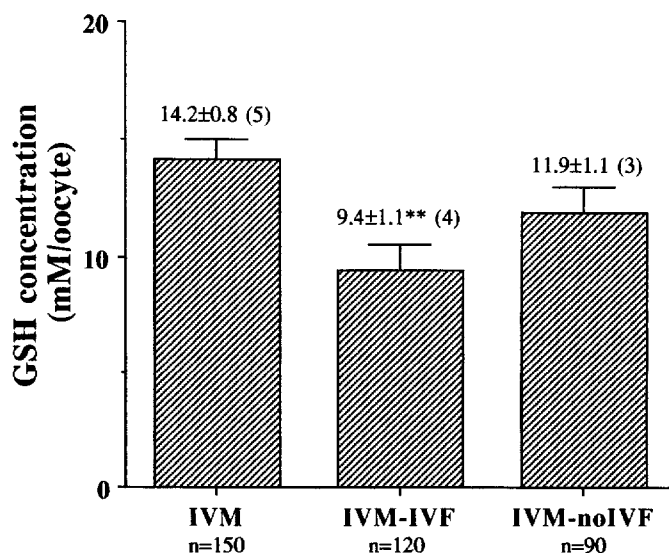

FIG. 5. Comparison of GSH concentration in pig oocytes matured in vitro (IVM), matured and fertilized in vitro (IVM-IVF) and matured but not inseminated in vitro (IVM-no IVF). The results represent the mean ± SEM of groups of oocytes collected in separate experiments. Data for IVM oocytes were obtained in experiment 3. Numbers in parentheses indicate number of replicates. n, total number of oocytes assayed. ** $p < 0.01$, compared with IVM oocytes.

in vitro ($p < 0.01$), and significantly lower in oocytes matured in vivo than in those matured in Waymouth medium in vitro ($p < 0.01$). However, the GSH concentration in oocytes matured in vivo did not differ from that of oocytes matured in mTLP supplemented with 0.14 or 0.57 mM CySH in vitro.

Experiment 5

The fifth experiment examined the GSH concentration in pig oocytes matured and fertilized in vitro (12 h after insemination). Oocytes were cultured for 36 h in mTLP supplemented with 0.14 mM CySH and transferred to fertilization medium with spermatozoa (IVM-IVF) or without spermatozoa (IVM-no IVF) before GSH concentration was assayed. As shown in Figure 5, the data obtained in this experiment compared favorably with that in pig oocytes matured for 36 h in vitro (IVM) obtained in experiment 3. GSH concentration was significantly lower in IVM-IVF oocytes than in IVM oocytes ($p < 0.01$).

DISCUSSION

The results of the study reported here show that 1) the composition of maturation medium affects the GSH concentration in pig oocytes; 2) the addition of CySH to maturation medium is associated with increased GSH synthesis by the pig oocyte, resulting in levels of GSH comparable to those found in oocytes matured in vivo; 3) GSH levels in pig oocytes change during oocyte maturation and after fertilization; and 4) GSH synthesis during oocyte maturation is an important factor for male pronucleus formation following fertilization.

The unpacking of sperm DNA during fertilization requires a reversal of at least some of the changes undergone by sperm nuclei during spermiogenesis and epididymal maturation: protamine disulfide bonds must be reduced and protamine must be removed [4, 16, 17]. Reduction of sperm nuclear disulfide bonds is the first step in the induction of sperm nuclear decondensation [18, 19]. Intracellular reducing power is generally maintained by GSH [1], and GSH could directly induce sperm nuclear decondensation in vitro [20, 21]. In this study, we have shown that pig oocytes matured in medium with as little as 0.04 mM CySH contained significantly more GSH, and hence had more disulfide-reducing power and exhibited a significantly increased rate of male pronucleus formation, than oocytes matured in the control medium. However, the rate of sperm nuclear decondensation, as estimated by the number of oocytes having enlarged sperm heads (the rate of sperm penetration), did not differ among those media. In general, male pronucleus formation is dependent on oocyte activation [19, 22–24], and sperm nuclei lacking disulfide bonds decondensed and transformed into pronuclei more rapidly than disulfide-rich ones [25]. Also, in our experience, the timing of sperm penetration into pig oocytes matured in

medium with CySH is essentially the same whether oocytes matured in medium with or without CySH (unpublished observation). We conclude that sufficient oocyte GSH is important to induce rapid and/or complete sperm decondensation in synchronization with oocyte activation, thus ensuring transformation of the fertilizing sperm head into the male pronucleus. In contrast, it appears that sperm decondensation may take place more slowly or incompletely in oocytes having insufficient GSH, resulting in asynchronous development of oocyte and sperm nuclei. Probably, at first, sufficient protamine disulfide bonds must be released, possibly through the action of GSH, such that oocyte cytoplasmic factors can gain access to the sperm chromatin, remove the protamine, and replace it with somatic histones as described by Perreault [5].

A previous study showed that both Waymouth medium and mTLP with 0.57 mM CySH (the same concentration as in Waymouth medium) were effective in promoting the ability of pig oocytes to form a male pronucleus [12]. The present results show that these media support a level of GSH synthesis during in vitro maturation of pig oocytes comparable to or higher than that found in in vivo matured oocytes. The limited capacity for male pronucleus formation in pig oocytes matured and penetrated by sperm in vitro and the high level of asynchronous development of male and female pronuclei have been described by several investigators [6–11]. Our finding that GSH concentrations declined when CySH is absent from the culture medium suggests again that CySH is a required external substrate for GSH synthesis in maturing pig oocytes.

It is well known that the ability of an oocyte to support sperm nuclear decondensation and male pronucleus formation depends upon the maturational state of the oocyte. In this study, we reported that GSH levels in pig oocytes changed during oocyte maturation and after fertilization, and that the addition of CySH to maturation media was effective for promoting the GSH synthesis in pig oocytes. These findings are consistent with the report of Perreault et al. [4] that GSH levels in hamster oocytes change during maturation and fertilization and the finding of Meister [26] that intracellular concentrations of GSH are dependent on the availability of CySH. Since the synthesis of GSH during in vitro maturation in pig oocytes requires exogenous compounds, the decline within 12 h in the GSH concentrations of oocytes cultured in medium without CySH could reflect a low rate of GSH synthesis, relative to the rate of GSH expenditure. In addition, GSH concentrations were significantly lower in oocytes penetrated by sperm than in controls (experiment 5). In theory, this reduction could have resulted from the block of GSH synthesis induced by sperm penetration or simply reflect use of some of the GSH to decondense penetrated spermatozoa. If either of these were the case it may be possible, given the high frequency of polyspermic fertilization in pig oocytes after in vitro fertiliza-

tion, to find an inverse relationship between GSH levels and number of decondensed sperm/oocyte.

On the other hand, GSH concentration was significantly higher in oocytes matured in mTCM-199 than that in oocytes matured in mTLP (Fig. 1). Since TCM-199 contains 0.06 mM cystine (the oxidized form of CySH) [27], it is possible that this cystine is converted into CySH by cumulus cells or oocytes and then incorporated into GSH. Conversion of cystine to CySH may also explain our finding that GSH synthesis was highest in oocytes matured in Waymouth medium, which contains both 0.06 mM cystine and 0.57 mM CySH [28]. Another possible explanation may be something else in these media involved in the control of GSH accumulation.

Pig oocytes contain unusually high levels of GSH compared with somatic cells (0.5–10 mM) [1, 2], or oocytes from other species, e.g., 8–10 mM in mouse and hamster oocytes [3–5]. Interspecies differences in oocyte GSH levels may correlate with differences in sperm chromatin stability, a function of the type of protamine present. It has been shown, for example, that species of sperm containing both protamines 1 and 2 (e.g., mouse and hamster) decondense faster after microinjection into hamster oocytes than those containing only protamine 1 (e.g., bull) [29]. Since boar sperm resemble bull sperm in that they contain only protamine 1 [30–32], perhaps they, too, are more stable and thus require more oocyte GSH for decondensation. This hypothesis remains to be tested.

In conclusion, our results indicate that the addition of CySH to the maturation medium is an important factor for GSH synthesis by the oocyte and enhances the ability of pig oocytes to form male pronuclei after sperm penetration. Additional studies are necessary to clarify the mechanism by which CySH is utilized by pig oocytes during maturation.

ACKNOWLEDGMENTS

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